

INVERTED REPEATS IN VIRAL GENOMES

M. Spanò, F. Lillo, S. Miccichè and R. N. Mantegna

*INFN Unità di Palermo and Dipartimento di Fisica e Tecnologie Relative
 viale delle Scienze, Edificio 18, I-90128, Palermo, Italy*

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We investigate 738 complete genomes of viruses to detect the presence of short inverted repeats. The number of inverted repeats found is compared with the prediction obtained for a Bernoullian and for a Markovian control model. We find as a statistical regularity that the number of observed inverted repeats is often greater than the one expected in terms of a Bernoullian or Markovian model in several of the viruses and in almost all those with a genome longer than 30,000 bp.

Keywords: Complex systems, Stochastic Processes, Viral Genomes, Secondary RNA structures, DNA probabilistic models.

1. Introduction

In the last few years there has been a progressively growing interest about the role of noncoding RNA (ncRNA) sequences producing functional RNA molecules having regulatory roles [1]. Prominent examples of these new regulatory RNA families are microRNA (miRNA) [2,3,4] and small interference RNA (siRNA) [5,6]. Most of these structures shares the property of being characterized by a hairpin secondary structure. DNA or RNA short sequences that may be associated to RNA secondary structures are present in genomes of different species of phages, viruses, bacteria and eukaryotes. Indication about the potential existence of RNA secondary structures can be inferred throughout the detection of short pair sequences having the characteristic of inverted repeats (IRs) in the investigated genomes [7].

In the present study we systematically investigate all the complete genomes of viruses publicly available at <http://www.ncbi.nlm.nih.gov/> on April 2003 to detect the presence of short IRs. The complete list containing the accession numbers of the investigated genome sequences is accessible at the web-page: <http://lagash.dft.unipa.it/viruses/List.txt>. The number of IRs found for different classes of structures and for each set of control parameters is compared with the prediction obtained for a Bernoullian (i.e. independent and identically distributed nucleotide

occurrence) and for a Markovian control models.

With this technique we are able to evaluate the presence of a large number of IRs that cannot be explained in terms of simple control models therefore indicating their potential biological role. For each virus, the study is performed – (i) over the entire genome and (ii) in its coding and noncoding regions.

2. Viral Genomes

During the past years about heigth hundred viral genomes have been completely sequenced. The complete sequence of their genomes is publicly accessible at specialized web pages. The database comprises different classes of viruses characterized by single stranded or double stranded nucleic acids, different infected organisms etc. In the present study we search the genomes for the presence of short subsequences, which might be associated with the existence of a secondary structure in regions of RNA originating from that subsequences.

Hairpin structures can occur when an IR is present in the nucleic acid sequence. For example, the DNA sequence 5’aGGAATCGATCTTaacgAAGATCGATTCCa3’ is a sequence having a sub-sequence GGAATCGATCTT which is the IR of AAGATCGATTCC. IRs of this type can form a hairpin having a stem of length 12 nucleotides and a loop (aacg) of length 4 nucleotide in the transcribed RNA. The number of IRs in complete genomes was first investigated with bioinformatics methods in long DNA sequences of eukaryotic (human and yeast) and bacterial (*E.coli*) DNA [7]. Successive studies have considered complete genomes such as the complete genomes of eubacterium *Haemophilus influenzae* [8], archaebacterium *Methanococcus jannaschii* and cyanobacterium *Synechocystis* sp. PCC6803 [9]. A Comparative genomic study of inverted repeats in prokaryotes has been investigated in Ref. [10].

An example of hairpin is shown in Fig. 1 for illustrative purposes. The figure caption describes in detail all the different part of such secondary structure. The example also presents a region of the stem with mismatches.

The simplest type of IR is the one without mismatches with the additional condition that the base pair before and after the stem are not complementary base pairs. Within this definition, Ref. [10] has shown that the number of IRs expected in a genome under the simplest assumption of a random Bernoullian DNA is given by the equation

$$n_{ex}(\ell, m) = N(1 - 2P_aP_t - 2P_cP_g)^2(2P_aP_t + 2P_cP_g)^\ell, \quad (1)$$

where N is the number of nucleotides in the genome sequence and P_a, P_c, P_g and P_t are the observed frequencies of nucleotides. Eq. (1) shows that the number of expected IRs is independent of m whereas it depends on the CG content of the genome. The CG content vary considerably across different genomes and for long genomes also across different regions of the same genome.

In the present study we are interested in a wider class of IRs in which the presence of mismatches is allowed, because these are present in many IRs with known biological role. Specifically, we detect all the IRs present in the complete genomes of viruses characterized by a stem length ℓ ranging from 6 to 20 and a loop length m ranging from 3 to 10. Inside the stem up to 2 mismatches are allowed provided that the number of links between complementary nucleotides inside the

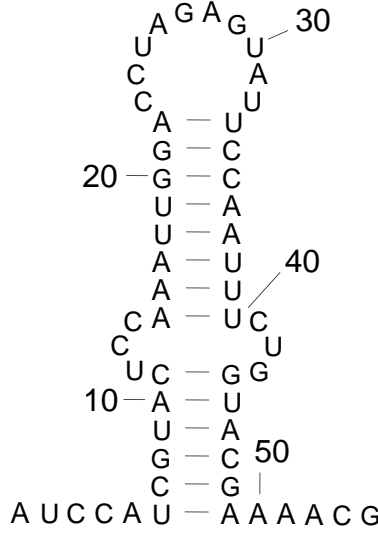


Fig 1. An example of the hairpin structure formed by a single stranded RNA. Bases from 6 to 22 constitute the left arm of the stem and bases from 33 to 49 constitute the right arm. The loop is made by bases 23 to 32. In this hairpin there is also a three base mismatch at bases 12-14 and 41-43. In the terminology used in the paper the parameter of this inverted repeat are $\ell = 17$, $m = 10$, and $mis = 3$.

stem is always equal or larger than 6. With these constraints and with the additional requirement of avoiding to count the same substructure as a portion of differently classified structures we focus on three different classes of IRs defined as follows. Example of the three different classes of inverted repeats detected are shown in the schematic drawing of Fig. 2. The first one is characterized by a stem with no mismatches with the additional check that the three base pairs before and after the stem are not complementary base pairs (see scheme at Fig. 2(a)). The second one is a stem with one mismatch inside and with the additional check that the two base pairs before and after the stem are not complementary base pairs (Fig. 2(b)). Finally the third one is a stem with two mismatches inside and with the additional condition that the base pair before and after the stem are not complementary base pairs (Fig. 2(c)). In Ref. [11] we generalize Eq. (1) for the three classes of structures considered here.

These equations allow therefore to perform a statistical test of the null hypothesis that the number of detected IRs is compatible with the assumption that viral genomes are Bernoullian symbolic sequences. This null hypothesis is equivalent to the assumption that IRs are observed in the genomes just by pure chance.

3. χ^2 tests

We perform several χ^2 test [12]. In all cases the χ^2 test is performed by comparing the number of IRs of the three classes described in Fig. 2 for different structures. Each structure is defined by a single value of ℓ ranging from 6 to 20. For each value of ℓ , the loop length m is varying from 3 to 10 and we verify the additional

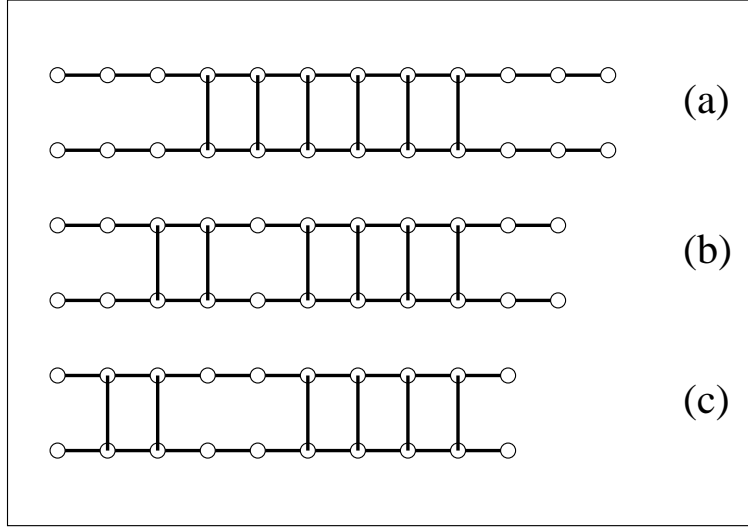


Fig 2. Schematic classification of the stem of the three classes of inverted repeats investigated in the present study. Each horizontal line indicates one of the RNA strands composing the stem of the potential secondary structure. A vertical line indicates the presence of a link between two complementary nucleotides whereas the absence of a vertical line indicates no complementarity. Structures of the kind (a) have not mismatches. Structures of the kind (b) have 1 mismatch and of kind (c) have 2 mismatches. They do not need to be contiguous.

condition of observing at least 6 links within the stem. When the expected number of IR of a structure defined as before is larger than 5 we consider the structure of that kind as a degree of freedom of the χ^2 test. When the number is smaller than 5 we aggregate different structures together until the number of expected IRs of the aggregated structures is larger than 5. In our procedure, the test cannot be performed for a certain number of viruses not reaching the threshold value of 5 for at least one type of the three investigated structures. Due to the wide range of lengths of the investigated genomes, the χ^2 test is realized with a variable number of degree of freedom. We set the confidence threshold of the χ^2 test at 0.05. This implies that the null hypothesis is rejected when the p-value is smaller than 0.05.

We have performed the χ^2 test of the hypothesis that genomes are described by a Bernoullian sequence on 736 genomes of viruses. In this set, 324 genomes pass the test whereas in the remaining 412 (56 %) the Bernoullian hypothesis must be rejected. In 409 cases out of 412 the statistical test is not passed due to an excess of the number of detected IRs.

Having verified that the null hypothesis of IRs compatible with a Bernoullian DNA sequence is falsified in a large number of viruses of our set, we have repeated the same test in the coding and non coding regions of the genomes separately. Specifically, we label as coding regions all the regions of viral nucleic acids that are annotated in the databases as sequences coding for aminoacids in proteins. We label as noncoding nucleic acid regions the remaining regions of the genomes, therefore including nucleic acid regions producing different kind of RNA.

When we investigate coding regions we are able to perform the test on 719

viruses. Within this set 356 (50 %) viruses do not pass the test whereas the remaining 363 do pass it. In all 356 cases the statistical test is not passed due to an excess of the number of detected IRs. Moving to the noncoding regions, the test can be performed on 540 viruses. The lower number is due to the fact that noncoding regions are typically just 10 percent of the viral genomes and therefore the number of expected IRs under the Bernoullian hypothesis is roughly a tenth of the number expected for the coding regions. Within this set of 540 viruses, 165 (31 %) do not pass the test whereas the remaining 375 do pass it. In 162 cases out of 165 the statistical test is not passed due to an excess of the number of detected IRs with respect to the expected ones in term of the Bernoullian hypothesis.

We have therefore verified that the Bernoullian hypothesis saying IRs are present in viral genomes just by chance is not passed in a significant fraction of the investigated viral genomes. Moreover, the tests performed separately in coding and noncoding regions show that the excess of IRs is observed both in coding and non-coding regions.

To shed light on the parameters influencing the validation or falsification of the null hypothesis in our viral database, we have investigated the number of viruses passing the Bernoullian test conditioned to the length of the viral genome. In this investigation we aim to check the characteristic of viruses in the entire genome and both in the coding and noncoding regions. For this reason we have selected the viruses where tests are possible both in the coding and in the noncoding regions. This set is composed of 524 viruses. Results are summarized in panel a) of Fig. 3. In the figure we show the value of the mean value $E[k|length]$ of the parameter k conditioned to the length of the viral genome. The parameter k assumes the value $k = 1$ if the genome passes the Bernoullian test or the value $k = 0$ in the opposite case. The three curves obtained respectively for the entire genome (triangles), its

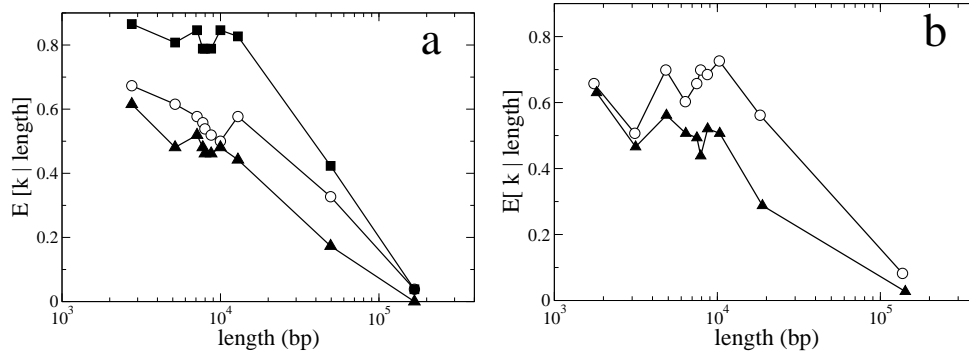


Fig 3. In panel a) it is shown the mean value of the parameter k describing if the viral genome passes the Bernoullian test ($k = 1$ when $p > 0.05$) or the opposite case ($k = 0$ when $p < 0.05$) conditioned to the length of the viral genome. The investigation is performed for a set of 524 viruses where the statistical test can be performed in the entire genome (triangles), its coding (circles) and noncoding (squares) regions. Analogously, panel b) shows the results of the same kind of analysis performed in the entire genome of 736 viruses under the assumption of a Bernoullian null hypothesis (triangles) and of 738 viruses under the assumption of a Markovian null hypothesis (circles). Each symbol groups the same number of viruses.

coding (circles) and noncoding (squares) regions share the same global behavior. The percentage of viruses passing the test is higher for shorter viruses and this percentage is steeply declining for length longer than 30,000 nucleotides. The conditional mean value reaches approximately the zero value for the longest genomes. The figure also shows that at fixed value of the genome length the test is passed with a higher percentage in noncoding than in coding regions. Global exceedence of the number of detected IRs with respect to the number of IRs expected in terms of a Bernoullian hypothesis is detected in a large number of viruses both in coding and in noncoding regions. The exceedence is progressively more pronounced in longer viral genomes.

The results we have obtained are not due to the fact that Bernoullian hypothesis is a zero-order null hypothesis not well reproducing the statistical properties of DNA and RNA sequences. To prove this sentence we have repeated the test by comparing the number of detected IRs in each complete genome with the number of IRs observed in a numerically simulated genome generated according to a 1-order Markov chain. The expected number of IRs is computed by simulating 100 different realizations of each genome with the same measured Markovian transition matrix. We have first measured the empirical Markovian transition matrix and therefore performed the test in all viral genomes. The χ^2 test with the Markovian hypothesis is performed on 738 viral genomes. Within this set of 738 viruses, 309 (42 %) do not pass the test whereas the remaining 429 do pass it. In 306 cases out of 309 the statistical test is not passed due to an excess of the number of detected IRs. This result shows that the Bernoullian assumption does not give results too different from the more accurate Markovian one. Panel b) of Fig. 3 shows the mean of the k parameter conditioned to the length of viral genomes for the Bernoullian and the Markovian hypotheses. The investigated sets comprises 736 viruses for the Bernoullian case and 738 for the Markovian one. In the rest of this paper we will present results obtained by using as a testing assumption the Bernoullian model. This choice is motivated by the fact that under the Bernoullian assumption we are able to use an analytical estimation of the expected number of IRs for each kind of the investigated structure whereas the expected number of IRs under the Markovian assumption can be obtained only on a statistical basis by performing numerical simulations.

The next step is then to investigate how uniform is the localization of each kind of structure in each virus with respect to the kind of considered coding or noncoding region. This new test is done separately for each structure identified as type a, b or c structure according to the classification of Fig. 2 and by its stem length ℓ . The null hypothesis is done in terms of a Bernoullian sequence to take advantage of the knowledge of analytical relations for the number of IRs expected for each structure. The test is devised to evaluate the degree of uniformity of the localization of observed structure of IRs inside each genome. The estimation of the number of expected IRs in coding $N_{uni}^{cr}(t, \ell)$ and noncoding $N_{uni}^{ncr}(t, \ell)$ regions under the assumption of not preferential (uniform) localization is done by using both the information about the total number of detected structures in the genome $N_{obs}(t, \ell)$ and the frequencies expected in terms of the Bernoullian hypothesis through the

equations

$$N_{uni}^{cr}(t, \ell) = N_{obs} \frac{N_{Ber}^{cr}(t, \ell)}{N_{Ber}^{cr}(t, \ell) + N_{Ber}^{ncr}(t, \ell)}, \quad (2)$$

and

$$N_{uni}^{ncr}(t, \ell) = N_{obs} \frac{N_{Ber}^{ncr}(t, \ell)}{N_{Ber}^{cr}(t, \ell) + N_{Ber}^{ncr}(t, \ell)}, \quad (3)$$

where t indicates the type of the structure (a, b or c) and $\ell \geq 6$ the stem length. These equations take into account the possibility that coding and noncoding regions might have different nucleotide frequencies.

We are able to perform the test in 1316 structures of 524 different viruses. Among these structures 1070 of 423 distinct viruses are consistent with the assumptions used in the test, which are (i) no preferential location between coding and noncoding and (ii) frequency of the IRs proportional to a Bernoullian expectation. Only in the remaining 246 (19 %) structures of 104 distinct viruses the statistical test is not passed and therefore in this restrict number of cases there might be a preferential location of these structure in one of the two consider regions. By looking at the specific contributions to the χ^2 test we note that in 204 cases of the 246 considered the detected structures are preferentially located in the noncoding regions. At first sight this result can be seen as not consistent with the results summarized in Fig. 3a where the Bernoullian test is more easily passed in noncoding rather than coding regions. But indeed there is no contradiction. In fact, among the 104 viruses having the 246 structures that do not pass the Bernoullian test and show a preferential location in the noncoding regions, 79 of them are longer than 10,000 bp. Moreover, these 79 viruses contains 220 of the 246 considered structures. For viruses of such length the difference between the conditional mean value of the test indicator k for coding and noncoding regions is much less pronounced than for viruses shorter than 10,000 bp and tend to disappear for longer viruses. The last χ^2 test is preferentially passed by structures located in longest viruses.

4. Conclusions

The present study has sistematically investigated the presence of IRs in 738 complete genomes of viruses. The investigated IRs can be both with a perfect matching of links within the stem and with the presence of mismatches up to a maximal value of 2. The empirically observed inverted repeats are compared with the values expected in terms of Bernoullian or Markovian model of genomes.

We find as a statistical regularity that the number of observed IRs are often greater than the one expected in terms of a Bernoullian or Markovian model in several of the viruses and in almost all those with a genome longer than 30,000 bp.

There is not a pronounced preferential location of these IRs in coding or noncoding regions in the majority of the considered viruses. This result is different from the one obtained by investigating complete genomes of bacteria [10] where a distinct preferential location of long structure of IRs in noncoding regions was observed.

We have therefore devised a methodology to detect sets of IRs whose existence cannot be explained in terms of simple random models of genome sequences. The selection of these sets of IRs may allow the future analysis of these secondary structure finalized to several distinct goals such as, for example, the detection of the

degree of homology among them both in terms of sequence similarity or in terms of free energy of the secondary structures.

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